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14. ABSTRACT
Proliferation and metastasis of many breast cancers depend on the activity of estrogen receptors (ERs). In addition to regulating nuclear gene expression, ERα can act in association with the plasma membrane. In vitro interaction studies by our laboratory led the hypothesis that membrane-localized ERα might weaken cell adhesions by impeding α/β catenin heterodimerization, which is necessary for the formation of stable adherens junctions. To test this hypothesis we constructed MCF7 lines that over express ERα variants known to alter ERα membrane location, and monitored the composition of adherens junctions in these cells. Consistent with a role of membrane-associated ERα in cell adhesion, these lines displayed distinct adhesion properties. However, we did not detect direct interactions of ERα with adherens junctions or ERα dependent changes in the composition of adherens junctions. In contrast to our hypothesis, membrane-associated ERα appeard to enhance cell adhesions. Interaction studies revealed that ERα preferentially interacts with α-catenin homo-dimers with the Arp2/3 complex resulting in changes in the actin polymerization. Analysis of MCF7 lines supported estrogen induced changes in actin polymerization in the vicinity of the membrane. These results suggest that membrane-localized ERα indeed play a role in the formation of cell adhesions, however likely through another mechanism as originally hypothesized.

15. SUBJECT TERMS- Estrogen receptor, non-nuclear activity, cell adhesions.

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Introduction

Proliferation and the formation of metastases by many breast cancers depend on the steroid hormone estrogen. The actions of estrogens are mediated by the estrogen receptors ERα and ERβ. These receptors function as hormone-regulated transcription factors that translate the presence of estrogen into changes in gene expression. New findings suggest that these receptors can also act outside of the nucleus and are often found associated with the plasma membrane. For example, it has been shown that proliferation of the human breast cancer cell line MCF7 depends on the interaction of cytosolic ERα with the Rous sarcoma virus kinase (Src) and the phosphatidyl inositol 3-kinase (PI3K) (1).

In contrast to their roles in regulating cell proliferation, very little is known about the mechanisms by which estrogens promote the formation of metastases. It has been suggested that estrogens aid this process by changing the expression of cell adhesion proteins, such as the transmembrane E-cadherin, which mediates cell-cell interactions (2). Results in our laboratory have opened the possibility for a different mechanism. While studying the functional interactions of ERα with other gene regulatory proteins, we found that ERα interacts with α- and β-catenin (Fig. 1). These proteins form a heterocomplex that interacts with the cytosolic domain of E-cadherin and stabilize cell-cell interactions by regulating the formation of the actin cytoskeleton at the plasma membrane. By mapping the interaction of ERα with α- and β-catenin, we identified that ER binds close to the structurally characterized heterodimerization domains of α- and β-catenin (3). Moreover, studies by others have demonstrated that ERα is present at the cell membrane of MCF7 cells, a tumorigenic mammary epithelial cell line, whose cellular adhesions are remodeled by estrogens (4,5). **Based on these findings we hypothesized that disruption of adherens junctions by estrogens involves the interaction of ERα with α- and β-catenin.** If true, this would represent a novel example of a non-nuclear activity of the estrogen receptor and steer ongoing studies on the role of estrogens in the remodeling of cellular adhesions into a new direction.

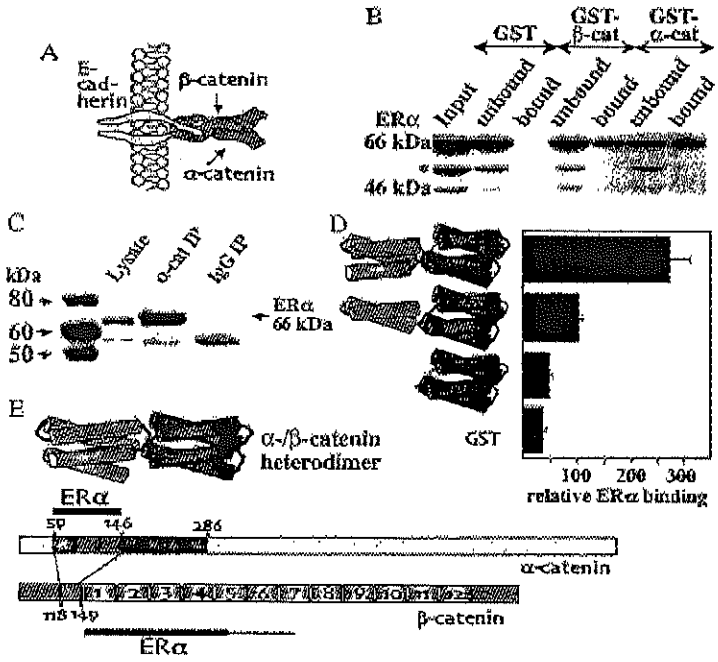

Fig. 1 Interaction of ERα with α- and β-catenin
A. Composition of E-cadherin-dependent adherens junctions. B. Interaction of ERα (66 kDa and 46 kDa isoforms) with recombinantly expressed and purified glutathione S-transferase β-catenin and α-catenin (amino acids 90-286) fusion proteins bound to glutathione agarose. Bound ERα was monitored by immunoblot analysis (* unspecific band recognized by the ERα antibody). These interactions are not hormone-dependent (data not shown). C. Co-immunoprecipitation of endogenous ERα 66 kDa and α-catenin in MCF7 lysates. D. GST pulldown assay monitoring the interaction of *in vitro* expressed and ^{35}S-labeled ERα (66 kDa) and GST fusions of the α-catenin fragments 55-286, 90-286 and 146-286. E. Map of the interactions of ERα with α- and β-catenin. ERα binds to the α-/β-catenin heterodimerization domain.

Research Accomplishments

Objective 1

Is membrane localization and interaction of ERα with α- and β-catenin necessary for the ability of 17β-estradiol (E2) to disrupt adherens junctions in MCF7 cells?

To investigate whether changes in the expression of ERα alter MCF7 cell-cell interactions, we stably integrated expression vectors for either ERα, ERα 46 kDa or the ERα mutant S518A (S518 is the human homologous residue to mouse S522) into MCF cells. Due to an alternative start site, ERα is expressed as a 66 kDa and 46 kDa form that lacks the N-terminal activation domain (AF1) (6). Through heterodimerization, ERα 46 kDa suppresses the transcriptional the transcriptional activity of ERα 66 kDa (6). Moreover, the N-terminus of the 46 kDa variant can be lipid-modified resulting in an enhanced recruitment of ERα 46 kDa to the plasma membrane (7). In contrast to ERα 46 kDa, the transcriptional response of the ERα mutant S518A has been shown to be comparable to that of ERα 66 kDa (8). However, this mutant has a dominant-negative effect on the membrane localization of ERα.

Construction and initial characterization of MCF7 lines - We completed the cloning and initial expression tests of the integration expression vectors for these various constructs. For each construct, we characterized 10 lines with respect to their cell morphology, growth, expression of the transgene and hormone responsiveness. Figure 2 shows examples of the characterizations for those lines that were finally selected. We also attempted the integration of the α-catenin 90-286, and β-catenin 141-286 fragments, which interact with ERα (Fig. 2). These fragments contain the α-/β-heterodimerization surface that mediates the interaction of α- and β-catenin in adherens junction complexes. However, MCF lines that overexpress α-catenin 90-286 or β-catenin 141-286 were heterogeneous, growth impaired and tend to apoptose suggesting that these fragments likely affect several aspects of β-catenin signaling. Therefore, we decided not to continue with these fragments.

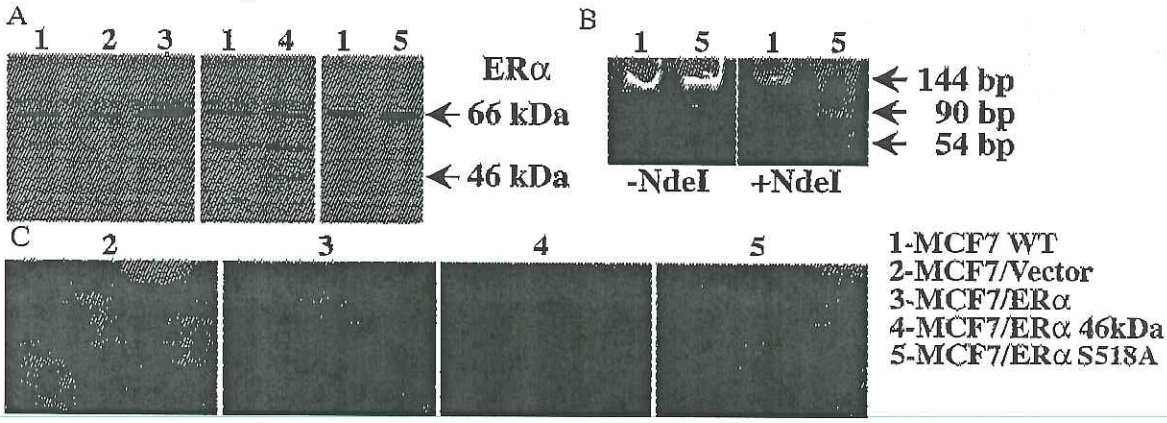

Fig. 2 Basic characterization of MCF7 lines
A. Expression of the transgenes monitored by immunoblot analysis using an ERα specific antibody. B. Since WT ERα and the ERα S518A mutant have similar molecular weights, expression of the mutant was monitored by cleaving amplified ERα cDNA with a restriction enzyme that only recognizes ERα S518A DNA. C. ERα expression in these MCF7 lines monitored by indirect immunofluorescence. Like in MCF7 WT, in the stable MCF7 lines the majority of ERα is nuclear.

Formation of cell adhesions by the MCF7 lines - MCF7 lines containing either an empty expression vector or vectors for ERα, ERα 46 kDa or the ERα mutant S518A displayed striking differences in their growth behavior (Fig. 3). While the MCF7/vector cells grew similar to WT MCF7 cells, MCF7/ERα and MCF7/ERα 46 kDa lines tended to form tight clusters. MCF7/ERα 46 kDa cells grew primarily as a monolayer, whereas MCF7/ERα 66 kDa cells formed multilayered clusters similar to those found in WT MCF7 cells treated with 17β-estradiol (E2). In contrast, MCF7/ERα S518A cells were only loosely associated with one another and apoptosed upon reaching confluency. Exposure to E2 for 5 days did not significantly alter the adhesive properties of the ERα 66 kDa, ERα 46 kDa and ERα S518A lines.

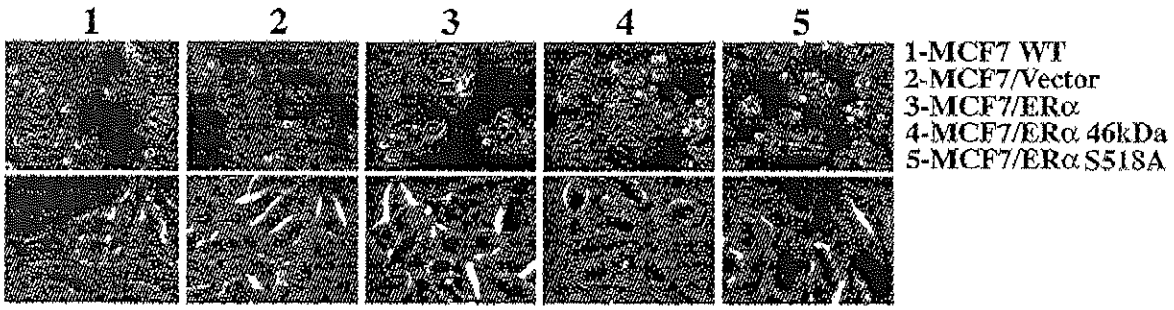

Fig. 3 Subconfluent growth pattern of MCF7 lines containing either an empty expression vector or vectors for ERα, ERα 46 kDa or the ERα mutant S518A.

ERα membrane localization - To monitor the membrane localization of ERα in MCF7 cells, we developed an experimental strategy to reliably prepare membranes from MCF7 cells without contaminations by cytosolic proteins (Fig. 4A). MCF7 fractionation experiments performed according to this protocol revealed that in MCF7 WT cells about 5% of total ERα is associated with membranes (Fig. 4B). Although in the presence of E2 expression of was ERα was generally reduced, the ratio of total to membrane-associated ERα remained unchanged (Fig. 4B). Repeat of these experiments with the MCF7 cell lines that contain either an empty expression vector, ERα or ERα 46 kDa gave similar results. However, in lines expressing ERα or ERα 46 kDa, ERα expression levels were generally higher compared to lines expressing endogenous ERα (Fig. 2A). Consistent with the indirect immunofluorescence analyses shown in figure 2C, the ERα membrane fraction of MCF7 lines expressing the ERα S518A mutant was reduced by about 30%. Together with the adhesive properties of these lines, these results suggested that membrane-localized ERα might be beneficial for the formation of stable junctions, which is in disagreement with our initial hypothesis.

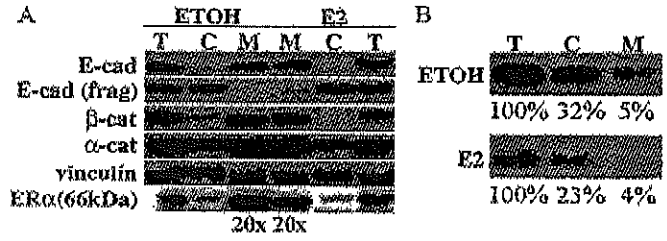

Fig. 4 Membrane localization of ERα in MCF7
A. Abundance of ERα and adherens junction proteins in total lysate (T), or cytosol (C) and membrane (M) fractions of MCF7 cells that had been treated with either vehicle (ETOH) or 1 nM E2 for 3 days. Proteins were identified by immunoblot analysis. Please note that compared to total cell lysate or cytosolic cell fractions the membrane fractions are 20-fold more concentrated. B. Quantitative analysis ERα (66 kDa) in cytosol and membrane fractions of MCF7 cells. Cells were treated as in A.

Interaction of ERα with E-cadherin adherens junction complexes in MCF7 cells - To determine whether ERα interacts with E-cadherin adherens junction complexes at the plasma membrane of MCF7 cells, we monitored the cellular localization of ERα, E-cadherin and β-catenin using indirect immunofluorescence. In the absence of E2, staining for E-cadherin/β-catenin and ERα did not overlap, suggesting that ERα does not interact with these junctions (Fig. 5). Treatment of MCF7 cells with E2 for three days resulted in a dispersion of junction complexes and an increase in ERα and E-cadherin/β-catenin co-localization. However, due to the disperse pattern of ERα and E-cadherin/β-catenin, overlap in the staining for these proteins is likely fortuitous.

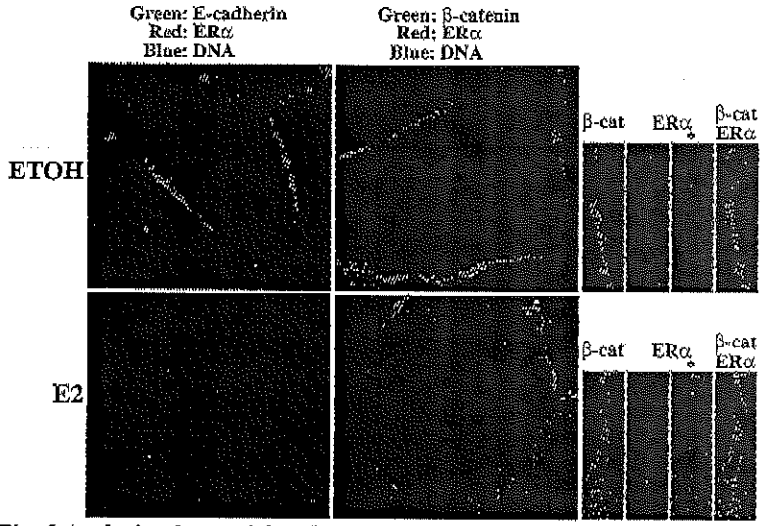

Fig. 5 Analysis of potential co-localization of ERα and E-cadherin/ β-catenin in MCF7 cells by indirect immunofluorescence. MCF7 cells were treated with either vehicle (ETOH) or 1 nM E2 for four days. Antibodies were fluorescent labeled as indicated. DNA is DAPI-stained. For a membrane fraction of vehicle and E2-treated cells β-catenin, ERα and β-catenin/ERα staining is shown separately. "*", enhanced images.

Thus far, we have been unable to detect ERα in co-immunoprecipitated E-cadherin adherens junction complexes (Fig. 6). Repeating these studies with the stable MCF7 lines shown in figure 3 yielded similar results.

In summary, contrary to our initial hypothesis, the results of objective 1 suggested that membrane-located ERα may rather stabilize than destabilize the formation of cell-cell interactions. Moreover, our findings did not support a direct interaction of ERα with adherens junctions.

Objective 2

Does E2 remodel adherens junctions by interfering with the interactions between α- and β-catenin? Does binding of ERα to α- and β-catenin impair the formation of the α- and β-catenin heterocomplex?

Our initial interaction studies revealed that ERα interacts with the α-/β-catenin heterodimerization domain suggesting that ERα might affect the formation of E-cadherin adherens junctions by regulating α-/β-catenin heterodimerization. Support for this model was provided by observations in endothelial cells where E2 seems to transiently impair the interaction between α- and β-catenin (9). To determine whether E2 influences the interactions between α- and β-catenin in MCF7 cells, we co-immunoprecipitated E-cadherin, α- and β-catenin form MCF7 cells that were treated with either vehicle of E2 for various times. The results from these studies, however, did not reveal any significant effect of E2 on the composition of E-cadherin complexes.

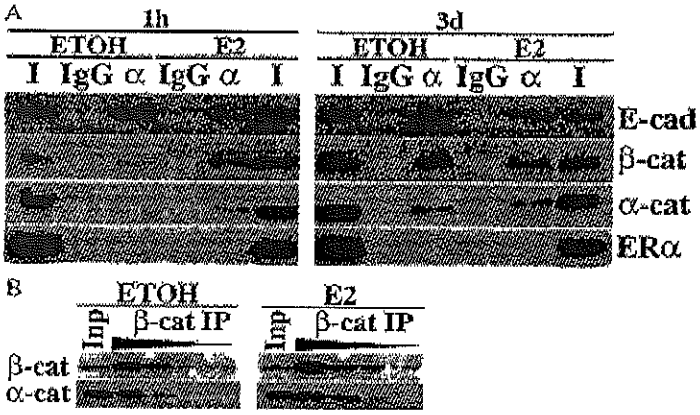

Fig. 6 Analysis of E-cadherin-dependent adherens junctions in the absence and presence of E2.
A. MCF7 cells were treated with either vehicle (ETOH) or 1 nM E2 for 1 or 3 days. E-cadherin-dependent adherens junctions were immunoprecipitated and probed with antibodies against E-cadherin, β-catenin, α-catenin and ERα. I, input; IgG, non-specific antibody, α, E-cadherin-specific antibody. B. Co-immunoprecipitation of α-/β-catenin in the presence of various concentrations of MCF7 cell lysates. MCF7 cells were treated with either vehicle or 1 nM E2 for 1h. Proteins were immunoprecipitated with a β-catenin-specific antibody. Immunoprecipitated complexes were probed with antibodies against α-catenin and β-catenin.

ERα interacts with α-catenin homodimers - Structural and biochemical studies have shown that α-catenin homodimerization and α-/β-catenin heterodimerization compete with each other (3). In both cases dimerization is mediated by the formation of a four-helix bundle (3). In the case of the α-catenin homodimer this bundle is formed by the α-catenin α-helices H1 and H2 (amino acids 90-146), whereas in the case of the α-/β-heterodimer the α-catenin α-helices H0, H1 and H2 (amino acids 59-146) are complemented by a β-catenin α-helix (amino acids 118-149) (Fig. 7A). To determine whether ERα interacts with the α-catenin homodimer or the α-/β-heterodimer, we purified these dimers and monitored their interaction with *in vitro* expressed ERα. As shown in figure 7A, ERα interacted preferably with the α-catenin homodimer.

A role of ERα in F-actin cytoskeleton remodeling? - Until very recently it was believed that the role of α-catenin in adherens junctions is to link E-cadherin/β-catenin to the cytoskeleton through direct or indirect interactions with F-actin. However, new results demonstrated that adherens junction complexes are not directly connected to the cytoskeleton. Instead these junctions appear to compete with the Arp2/3 complex for binding to α-catenin, which results in a switch from branched F-actin fibers to parallel F-actin fiber bundles that stabilize cell-cell interactions (Fig. 7B) (11). This model opens the possibility that the interaction of ERα with α-catenin homodimers interfers with the α-catenin/Arp2/3 interaction. To test whether this might be the case, we phalloidin-stained the F-actin cytoskeleton in MCF7 cells that were treated with either vehicle or E2 for 4 days. Consistent with an effect of E2 on F-actin polymerization, in E2-treated cells par-

allel F-actin fibers were frequently disrupted and branched F-actin fibers appear to be more frequent (Fig. 7C). To complete this study for publication, presently we are developing an *in vitro* assay to monitor the effect of ERα on the interaction of α-catenin homodimers with the Arp2/3 complex.

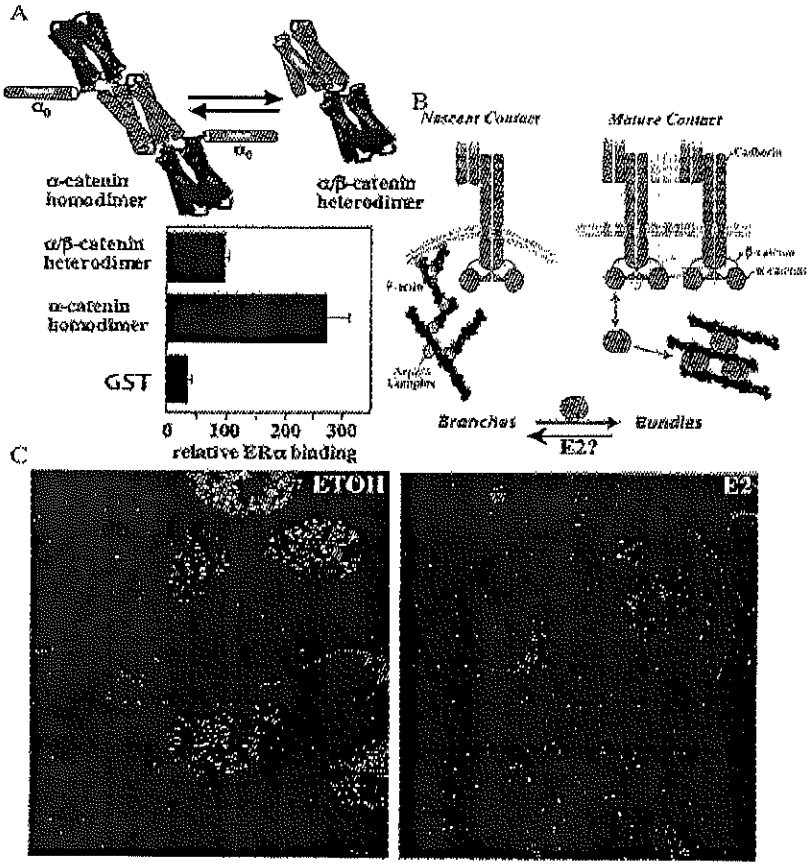

Fig. 7 Role of E-cadherin-dependent adherens junctions in F-actin remodeling
A. GST-pulldown experiment comparing the interaction of ERα with GST or GST-fused α-catenin homodimers and α-/β-catenin heterodimers. the α-helix of β-catenin that interacts with α-catenin is shown in pink. B. Model for the adherens junction-mediated changes in F-actin polymerization according to (11). A high concentration of adherens junctions establishes a local equilibrium between α-catenin homodimers and α-/β-catenin heterodimers. The α-catenin homodimers bind F-actin and prevent the branching of F-actin fibers by the Arp2/3 complex. C. ERα and F-actin localization in MCF7 cells that have been treated with either vehicle (ETOH) or 1 nM E2 for four days. F-actin was stained with phalloidin, ERα by indirect immunofluorescence using an ERα-specific antibody.

Key Research Accomplishments

Objective 1:
Is membrane localization and interaction of ERα with α- and β-catenin necessary for the ability of 17β-estradiol (E2) to disrupt adherens junctions in MCF7 cells? (Total: 9 months)

Task 1: Stable integration of ERα S518A (mouse ERα S522A), α-catenin 90-286 and β-catenin 141-286 into MCF7 cells

- **We successfully constructed MCF7 lines containing either an empty expression vector or integrated expression vectors for ERα, ERα 46 kDa or the ERα mutant S518A and completed the initial characterization of these lines.**
- **We also constructed lines that overexpress α-catenin 90-286 or β-catenin 141-286. However, these lines were heterogeneous and growth-impaired indicating that these fragments affect other aspects of β-catenin signaling. Therefore, we decided to not continue the analysis of these lines.**

Task 2: Does expression of ERα, ERα S518A, α-catenin 90-286, and β-catenin 141-286 affect ERα membrane localization and the ability of 17β-estradiol (E2) to impair adherens junction formation in MCF7 cells?

- **We established a protocol for the quantitative analysis of ERα membrane localization in MCF7 cells and monitored ERα membrane localization in the constructed MCF7 lines in the absence and presence of E2. In MCF7 cells, as well as in lines containing either the integrated expression vector, ERα or ERα 46 kDa about of 5% of ERα was associated with membranes. This fraction did not change upon treatment of these cells with E2. However, in the ERα and ERα 46 kDa lines ERα levels were generally higher compared to cells expressing endogenous ERα. Consistent with reports in the literature, in lines expressing the ERα S518A mutant, the fraction of membrane-associated ERα was about 30% lower than in the other lines.**
- **MCF lines overexpressing ERα or ERα 46 kDA displayed stronger and MCF7 lines expressing ERα S518A weaker cell-cell adhesions than MCF7 lines expressing endogenous levels of ERα. In contrast to our in itial hypothesis, these results indicate that membrane-associated ERα is beneficial for the formation of stable cell-cell interactions.**

Objective 2:
Does E2 remodel adherens junctions by interfering with the interactions between α- and β-catenin? Does binding of ERα to α- and β-catenin impair the formation of the α- and β-catenin heterocomplex? (Total: 3 months)

- **We successfully developed a co-immunoprecipitation protocol for the quantitative analysis of E-cadherin-dependent adherens junctions in MCF7 cells.**
- **Co-immunoprecipitation and *in vivo* co-localization studies did not provide any evidence for a direct association of ERα with adherens junctions or for E2-dependent changes in the interactions between E-cadherin, α-catenin and β-catenin. These results argue against the hypothesis that the interaction of ERα with α-catenin and β-catenin interferes with the formation of the α-/β-catenin heterodimer.**

Task 4: Determine whether ERα and β-catenin compete for binding to α-catenin using *in vitro* interaction measurements.

- **By comparing the interactions of ERα with the purified α-/β-catenin heterodimer and α-catenin homodimer *in vitro*, we found that ERα interacts preferably with the α-catenin homodimer.**
- **α-/β-catenin heterodimerization induces the formation of parallel, junction-stabilizing F-actin bundles, by disrupting the interaction of the α-catenin homodimer with the Arp2/3 complex. Consistent with a role of E2 in the regulation of the actin cytoskeleton, we found that E2-treated MCF7 cells have significant fewer parallel F-actin bundles than untreated cells. Hence, our new hypothesis is that membrane-associated ERα stabilizes junction formation by weakening the interaction of the α-catenin homodimer with the Arp2/3 complex. We are in the process of testing this hypothesis by monitoring the interactions between ERα, α-catenin and Arp2/3 *in vitro*.**
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Reportable Outcomes

Abstract:
J. Jacobson, L. Schwarcz, M. Lib-Myagkov, and Beatrice Darimont,
Regulation of cell adhesions by estrogen receptor alpha
Gordon Conference "Signaling by Adhesion Receptors", South Hadley, MA
June 25-30, 2006

Poster Presentations:
J. Jacobson, L. Schwarcz, M. Lib-Myagkov, B. Darimont
Regulation of cell adhesions by estrogen in human MCF7 breast cancer cells
Westcoast "Comparative Endocrinology" Meeting, Newport, USA.
March 23-25, 2006

J. Jacobson, L. Schwarcz, M. Lib-Myagkov, B. Darimont
Regulation of cell adhesions by estrogen receptor alpha
Gordon Conference "Signaling by Adhesion Receptors", South Hadley, MA
June 25-30, 2006

Research Seminar:
J. Jacobson, M. Lib-Myagkov, L. Schwarcz, T. Takayama, B. Darimont
Linking steroid and beta-catenin signaling
Hospital of Special Surgery, Cornell University, New York
September 09, 2006

J. Jacobson, M. Lib-Myagkov, L. Schwarcz, T. Takayama, B. Darimont
Linking steroid and beta-catenin signaling
University of Indiana, Bloomington
May 29, 2007

Experience/Training:
Doctoral training: Jana Jacobson
Research experience: Sachiko Takayama, Lin Fang

Conclusions

Proliferation and the formation of metastases by many breast cancers depend on the steroid hormone estrogen (E2) whose actions are mediated by the estrogen receptors ERα and ERβ. Very little is known about the mechanisms by which estrogen promotes the formation of metastases. Results in our laboratory suggested the possibility that membrane-associated ERα might affect the formation of E-cadherin-dependent adherens junctions by regulating the interactions between β-catenin and α-catenin in these junctions. Consistent with this hypothesis, we found that in human MCF7 breast cancer cells ERα can be membrane-associated. Moreover, overexpression of ERα mutants that alter the association of ERα with membranes did affect the formation of cell adhesions. However, analysis of E-cadherin-dependent adherens junctions by indirect immunofluorescence and co-immunoprecipitation approaches failed to provide evidence for direct interactions of ERα with these junctions or for E2-dependent changes in the composition of these junctions. In contrast to our initial hypothesis, membrane-localization of ERα appeared to be beneficial for the formation of stable junctions. *In vitro* interaction studies revealed that ERα interacts preferentially with α-catenin homodimers, which through interactions with the Arp2/3 complex promote the formation of branched F-actin fibers that are typical for subconfluent cultures. While confluent MCF7 cultures showed primarily parallel F-actin bundles that are typical for mature adherens junctions, in E2-treated MCF7 cells branched F-actin fibers were enriched. These results suggest that membrane-associated ERα might stabilize cell-cell interactions by weakening the interaction of the α-catenin homodimer with the Arp2/3 complex. In summary, our results supported a role of membrane-localized ERα in the regulation of cell adhesion in breast cancer cells. However, the mechanism of this regulation is likely different than originally proposed.

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